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Complex biogeographic history of a Holarctic passerine

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Our analysis of the ND2 sequences revealed six clades within winter wrens (*Troglodytes troglodytes*). These clades corresponded to six geographical regions: western Nearctic, eastern Nearctic, eastern Asia, Nepal, Caucasus and Europe, and differed by 3–8.8% of sequence divergence. Differences among regions explained 96% of the sequence variation in winter wren. Differences among individuals within localities explained 3% of the sequence variation, and differences among localities within regions explained 1%. Grouping sequences into subspecies instead of localities did not change these proportions. Proliferation of the six clades coincided with Early and Middle Pleistocene glaciations. The distribution of winter wren clades can be explained by a series of five consecutive vicariant events. Western Nearctic wrens diverged from the Holarctic ancestor 1.6 Myr before the present time (MYBP). Eastern Nearctic and Palaeartic wrens diverged 1 MYBP. Eastern and western Palaeartic birds diverged 0.83 MYBP. Nepalese and east Asian wrens diverged 0.67 MYBP, and Caucasian birds diverged from European wrens 0.54 MYBP. The winter wren has a much greater degree of inter- and intracontinental differentiation than the three other Holarctic birds studied to date—dunlin (*Calidris alpina*), common raven (*Corvus corax*) and three-toed woodpecker (*Picoides trydactylus*)—and represents an example of cryptic speciation that has been overlooked.

Keywords: winter wren; biogeography; phylogeography; speciation; vicariance; Holarctic

1. INTRODUCTION

Very little is known about the phylogeographic structure of avian Holarctic species. Zink *et al.* (1995) surveyed 13 species inhabiting both Eurasia and North America and found that 11 species showed at least some degree of intra-specific differentiation in mitochondrial DNA (mtDNA) between continents. Only three studies addressed large-scale phylogeography of Holarctic avian taxa. Wenink *et al.* (1996) found three distinct mitochondrial clades of dunlin (*Calidris alpina*) corresponding to three large geographical regions: Europe, Canadian Arctic and Siberia/Alaska. The latter was subdivided into three subclades: west Siberian, east Siberian and Alaskan. The Canadian clade was the most distantly related to the other two clades and differed from them by 3.3% of uncorrected sequence divergence of the most variable domains, I and II of the mitochondrial control region (CRI and CRII; Wenink *et al.* 1996). Omland *et al.* (2000) found no differentiation in mtDNA between common ravens (*Corvus corax*) from Eurasia and North America. However, birds from western North America, south of the Canadian border, formed a distinct clade that was the sister to the Chi-

huahua raven (*C. cryptoleucus*), and was well differentiated from the Holarctic raven (2.69% of uncorrected divergence in the cytochrome *b* gene and 2.14% in CRI; calculated from Omland *et al.* (2000), Table 1). By contrast, mtDNA of three-toed woodpeckers (*Picoides tridactylus*) from Eurasia and North America differed by *ca.* 4% despite the lack of phylogenetic structure within continents (Zink *et al.* 2002). Both the common raven and the three-toed woodpecker are uniformly distributed across boreal regions of both continents.

Unlike the dunlin, which inhabits primarily arctic tundra, but similar to the common raven and three-toed woodpecker, the winter wren (*Troglodytes troglodytes*) is widely distributed across the temperate zone of the Holarctic. However, in both continents it is widely distributed in a north–south direction only in the eastern and western parts of the continents. In the middle of both continents, its distribution has large gaps. In the Nearctic, the eastern and western parts of the range are connected by a narrow strip of habitat extending across Manitoba, Saskatchewan and Alberta. In the Palaeartic, the eastern and western parts of the range are separated by a narrow gap between south-central and extreme eastern Turkmenistan (Brewer 2001).

This distribution pattern and sedentary/limited migration lifestyle of winter wren could result in genetic differentiation at different scales, namely within and between continents. The winter wren is one of the most complex and phenotypically diverse taxa. Brewer (2001)

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listed 39 subspecies, and noted that many more were described in the Nearctic. The majority of winter wren subspecific designations are based on slight variations of plumage coloration and intensity of underbody barring. Some geographically proximate subspecies of winter wren form groups that are distinguished by their appearance and vocalization. In the Nearctic, there are three phenotypically distinct groups: eastern, western and Alaska/Aleutian (Pyle 1997; National Geographic Society 1999). In the Palaearctic, eastern Asian winter wrens differ from European and western and central Asian birds by much darker brown plumage (Stepanyan 2003). Interestingly, birds from the western Nearctic and eastern Asia, and birds from the eastern Nearctic and Europe, are more similar in appearance to each other than to birds from opposite sides of their respective continents.

The complex geographical pattern of phenotypic variation has fostered much confusion about the evolutionary and biogeographic history of the winter wren. Although a generally accepted hypothesis suggests that the Palaearctic was colonized by the western Nearctic birds through the Bering Strait (Brewer 2001), alternative hypotheses were proposed by several authors. For example, Hejl *et al.* (2002) suggested separate invasions of the Palaearctic by both eastern and western Nearctic winter wrens (Hejl *et al.* 2002). Interestingly, all of these hypotheses are based on dispersal scenarios, and none considers vicariance. Some authors argue that because any distribution pattern can be explained by numerous, equally parsimonious dispersal hypotheses, vicariance should be ruled out first, and dispersal should be invoked when vicariance fails to explain observed distribution patterns (Ronquist 1997).

We present data on large-scale phylogeographic patterns in the Holarctic winter wren, and compare population genetics parameters among localities with relatively large sample sizes. We also discuss the historical biogeography of winter wren and use dispersal–vicariance analysis (DIVA; Ronquist 1996, 1997) to determine if vicariance alone is sufficient to explain the observed geographical structuring of winter wren haplotypes.

2. MATERIAL AND METHODS

A total of 97 winter wren samples were obtained from 24 localities across the Holarctic (figure 1). We also used 16 individuals of 14 wren species as outgroups (electronic Appendix A; available on The Royal Society's Publications Web site).

Total genomic DNA was extracted from frozen or 96% ethanol-preserved tissue samples using a QIAamp Tissue Kit (QIAGEN Inc.). Each individual complete mitochondrial ND2 gene (1041 bp) was amplified with Perkin-Elmer PCR reagents and primers L5215 (Hackett 1996) and H1064 (CTTTGA AGGCCTTCGGTTTA, designed by S.V.D.). The PCR fragments were sequenced directly on an ABI-3700 sequencer.

Sequences were aligned automatically by SEQUENCHER 3.1.1 (Gene Codes Corporation). The alignment did not require editing because there were no indels in the ND2 sequences. A maximum-likelihood (ML) phylogenetic analysis was performed using PAUP* (Swofford 1998). ML model and parameters were determined by the hierarchical likelihood ratio test (hLRT) in MODELTEST 3.06 (Posada & Crandall 1998). Taxa were added

randomly for both ML and its bootstrap analysis (100 replicates). Population genetics parameters were calculated in ARLEQUIN 2.000 (Schneider *et al.* 2000). DIVA (Ronquist 1996, 1997) with its default settings was used for reconstruction of the biogeographic history of winter wren.

3. RESULTS

(a) *Geographical structuring of winter wren haplotypes*

The initial phylogenetic analysis (results not shown) indicated that winter wrens are most closely related to marsh (*Cistothorus palustris*) and sedge (*C. platensis*) wrens than to the other outgroup species (electronic Appendix A), including two members of *Troglodytes*: northern house wren (*T. aedon*) and mountain wren (*T. solstitialis*). Therefore, we used marsh and sedge wrens as outgroups for the phylogenetic analysis of winter wren haplotypes.

Among 97 individuals of winter wren, 51 unique haplotypes were distinguished based on 126 variable sites (120 transitions and nine transversions). The hLRT selected the TrN+G model (Posada & Crandall 1998) for complete ND2 sequences of winter, marsh and sedge wrens. The TrN+G is a submodel of the general time reversible (GTR) model (Rodríguez *et al.* 1990) in which transversions are weighted equally and the discrete-gamma model of substitution rates across sites (Yang 1994) is included. Guanine was under-represented and cytosine was over-represented in ND2 sequences (A = 30.08%, C = 39.62%, G = 10.26%, T = 20.04%; *G*-test $p = 0.0110$). All taxa shared this nucleotide bias and there was no evidence of heterogeneity of base composition among taxa. GenBank accession numbers for ND2 sequences are AY460221–AY460333.

The ML analysis of wren haplotypes resulted in a single tree. The molecular clock assumption for this tree was not rejected ($-\ln L$ without molecular clock enforced = 3092.786 33, $-\ln L$ with molecular clock enforced = 3124.447 44; $-2\Delta\ln L = 63.322 22$, d.f. = 51, $p = 0.1154$). Winter wren haplotypes formed six geographically concordant clades (figure 2). These clades corresponded to six major geographical regions: Europe, Caucasus, Nepal (central Asia), eastern Asia, eastern Nearctic and western Nearctic (figure 1). Each clade was supported by bootstrap values of greater than or equal to 78%. The relationships among these clades, however, were not strongly supported except for the sister-group relationship of Caucasian and European clades, which received 94% bootstrap support (figure 2).

The Palaearctic clades were divided into two pairs of sister clades: the east Asian clade and a single haplotype from Nepal (3.7% ML divergence between clades excluding intraclade variation), and European and Caucasian clades (3.0% ML divergence). The ML divergence between these pairs was 4.6%. Palaearctic birds were more closely related to the eastern Nearctic clade (5.6% ML divergence) than to the western Nearctic clade (8.8% ML divergence), so the two Nearctic clades were not sisters. Conversion of the node depths (excluding intraclade variation; figure 2) into million years before the present time (MYBP) using an ND2 molecular clock calibration for

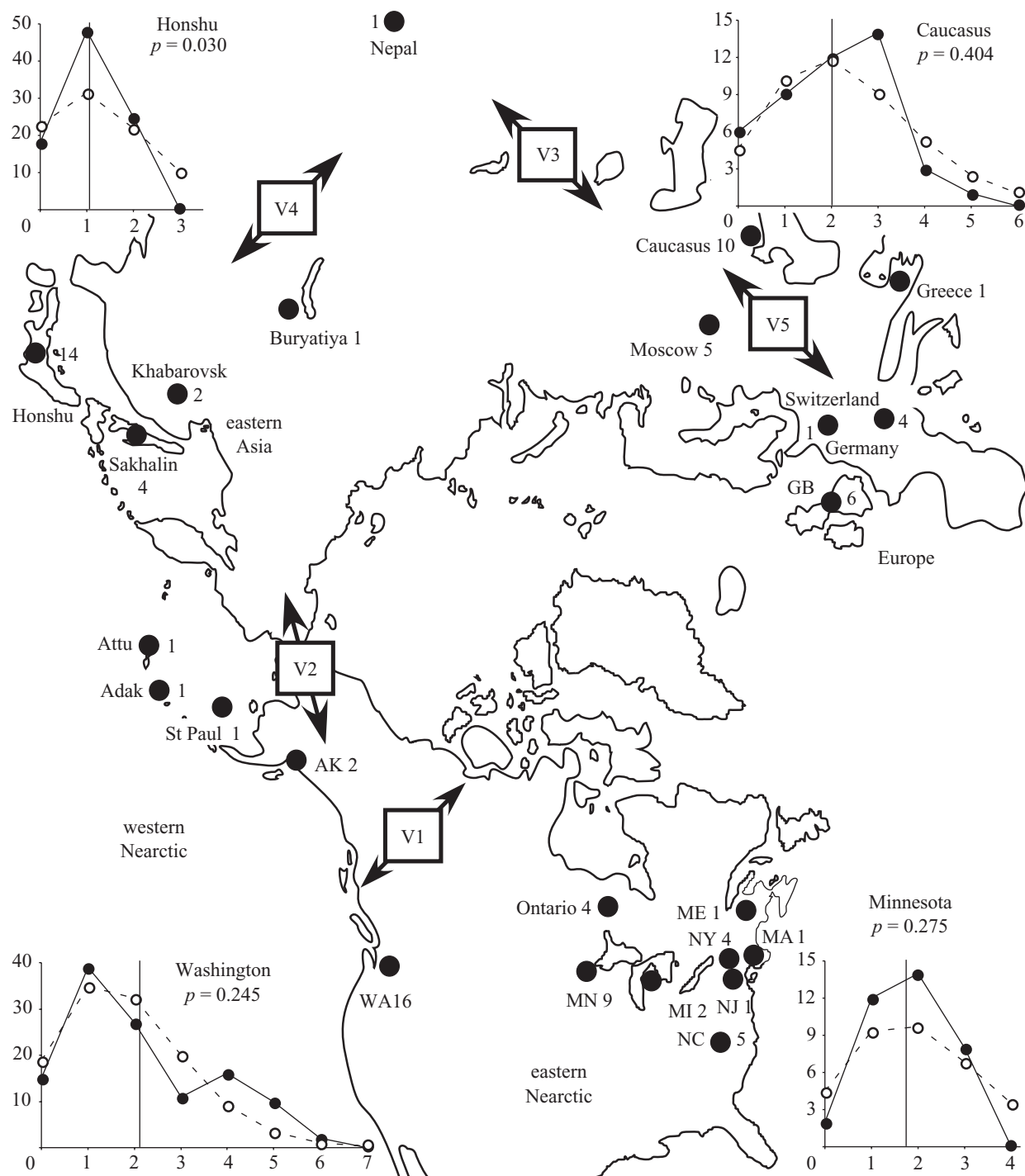


Figure 1. Geographical regions, localities, sample sizes and mismatch distributions. Vicariant events are labelled with the letter 'V' and their consecutive number. Mismatch distributions: *x*-axis, number of pairwise differences; *y*-axis, observations. Solid lines, observed distributions; dashed lines, expected distributions; vertical lines, mean mismatch. The *p*-values are for the differences from expectation of sudden population expansion.

Galapagos mocking birds (0.0276 substitutions per site per lineage Myr⁻¹; B. S. Arbogast, P. T. Boag, G. Seutin, P. R. Grant, B. R. Grant, S. V. Drovetski, R. Curry and D. J. Anderson, unpublished data) indicated that winter wrens radiated into the six lineages in Early–Middle Pleistocene. The western Nearctic clade diverged from the common ancestor of the other winter wren clades first, *ca.* 1.6 MYBP. The eastern Nearctic birds diverged from the common ancestor of the Palearctic clades *ca.* 1 MYBP.

Eastern and western Palearctic winter wrens diverged 0.83 MYBP. East Asian birds and Himalayan birds diverged 0.67 MYBP, and Caucasian and European birds diverged last, 0.54 MYBP. Although sedge and marsh wrens may not be the closest relatives of the winter wren, divergence between the common ancestors of these two lineages occurred 13 MYBP. Therefore, winter wren could have existed since the Middle Miocene, long before the radiation of the six current clades.

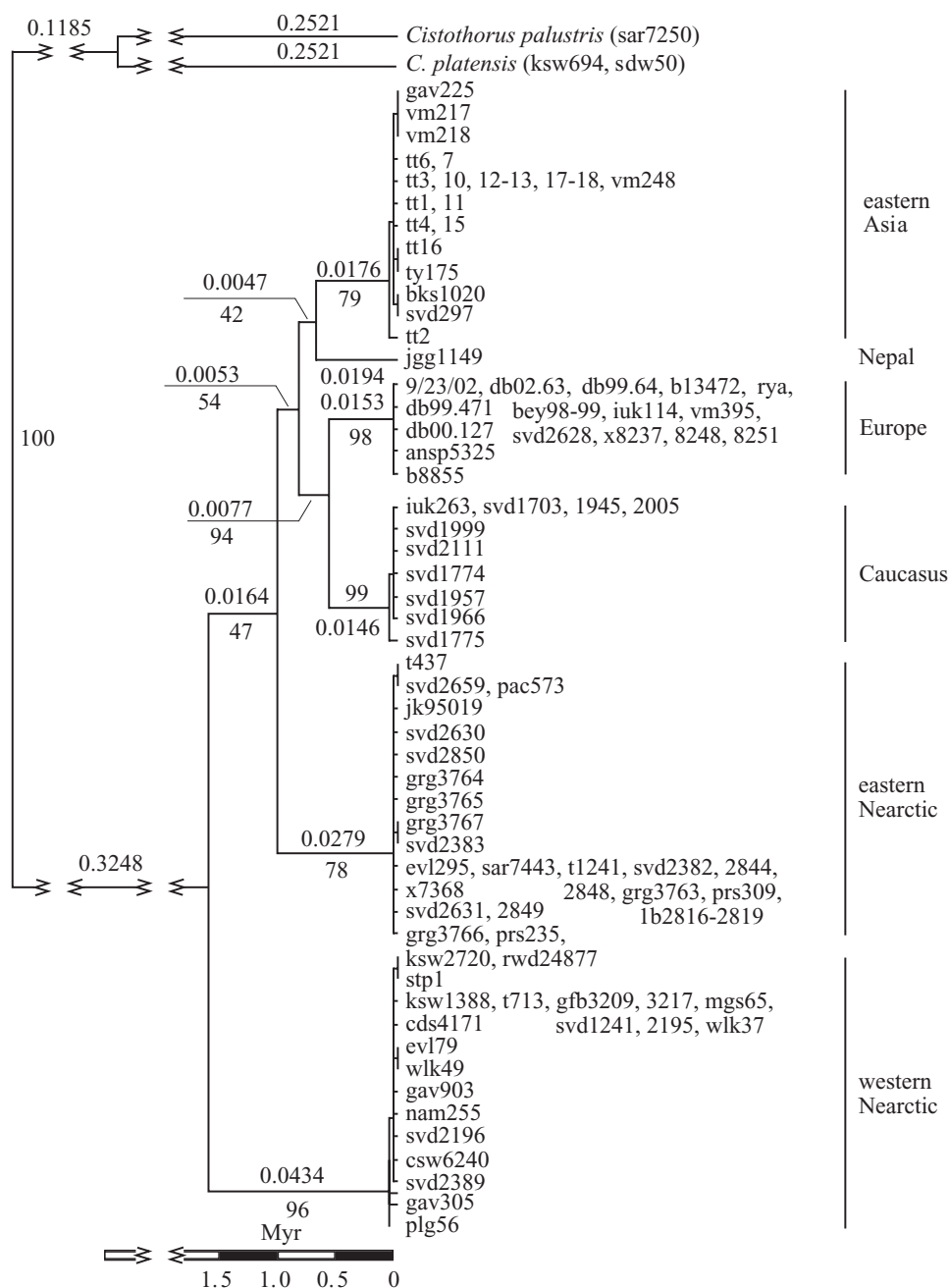


Figure 2. Maximum-likelihood tree of unique haplotypes. Sample numbers are listed at the tips (for localities and subspecies see electronic Appendix A). Numbers next to branches indicate ML bootstrap values (100 replicates with random addition of taxa) and branch lengths (substitution/site).

Using PAUP* we calculated average ML pairwise differences among haplotypes within clades and their standard deviations (\pm s.d.). They were $0.34 \pm 0.16\%$ for the western Nearctic, $0.26 \pm 0.11\%$ for the eastern Nearctic, $0.30 \pm 0.14\%$ for eastern Asia, $0.20 \pm 0.08\%$ for Europe and $0.27 \pm 0.11\%$ for Caucasus.

Analysis of molecular variance (AMOVA; Excoffier *et al.* 1992) indicated that most of the sequence variation was due to differences among the six geographical regions. Differences among individuals within populations accounted for 3% of the sequence variation, differences among localities within regions accounted for 1% of the sequence variation, and differences among geographical regions accounted for 96% of the sequence variation.

Grouping of haplotypes by subspecies rather than by localities did not change the AMOVA results.

(b) Population genetics and demography of the winter wren

Only one of the 11 localities with a sample size of four or more had a marginally significant Tajima's *D*-value (Tajima 1989) indicating that the selective neutrality assumption is appropriate for the evolution of ND2 sequences of winter wren (table 1).

The ML nucleotide diversity varied from 0 to 0.0029 (table 1). All European populations had very low nucleotide diversities. British wrens were more genetically diverse than birds from continental Europe, where 10

Table 1. Tajima's p -values, age, nucleotide diversity based on ML (π_n) and on infinite site model (π_n), and their standard deviations (s.d.) for populations with $n \geq 4$.

locality	n	Tajima's		ML π_n	s.d.	π_n	s.d.
		p	age (MYBP)				
WA	16	0.047	0.038	0.002 104	0.001 642	0.002 017	0.001 330
MN	9	0.248	0.032	0.001 771	0.000 885	0.001 708	0.001 233
NY	4	0.284	0.018	0.000 968	0.000 617	0.000 961	0.000 952
Ontario	4	1.000	0.000	0.000 000	0.000 000	0.000 000	0.000 000
NC	5	0.079	0.051	0.002 835	0.001 383	0.002 690	0.001 986
Sakhalin Is.	4	0.739	0.052	0.002 870	0.001 560	0.002 722	0.002 147
Japan	14	0.177	0.019	0.001 041	0.000 669	0.001 035	0.000 813
GB	6	0.089	0.024	0.001 308	0.000 968	0.001 281	0.001 059
Moscow	5	1.000	0.000	0.000 000	0.000 000	0.000 000	0.000 000
Switzerland	4	1.000	0.000	0.000 000	0.000 000	0.000 000	0.000 000
Caucasus	10	0.266	0.037	0.002 043	0.001 264	0.001 964	0.001 358

birds from Switzerland, Germany and the Moscow region shared a single haplotype. An application of the ND2 molecular clock indicates that all localities, except the three localities covered by ice sheets (Ontario, Moscow and Switzerland), were colonized by winter wrens before or during the Last Glacial Cold Stage 0.021–0.017 MYBP (Adams 2002). The three glaciated localities had all individuals sharing the same haplotype (figure 2), indicating a very recent colonization of these areas by winter wrens.

The nucleotide diversity based on infinite site model (π_n) was slightly lower than nucleotide diversity based on ML corrected distances (ML π_n). However, the values were strongly correlated ($Y = 1.733 \times 10^{-5} + 0.950 \times X$; d.f. = 10, $r^2 = 1$, $p < 0.0001$).

Mismatch distributions were calculated for four localities that had a sample size of nine or more (Japan, Caucasus, Minnesota and Washington; figure 1). All four localities were at similar latitudes 43°–48° N. Mismatch distributions for Caucasus, Minnesota and Washington had similar mean mismatch values and did not differ significantly from the expectation of recent demographic expansion (Slatkin & Hudson 1991; Rogers & Harpending 1992). By contrast, the mismatch distribution for the Honshu sample differed significantly from that expected, despite its apparent unimodality and its mean mismatch value, which was half of each of the other three sample values.

(c) *Dispersal–vicariance analysis and area relationships*

DIVA (Ronquist 1996, 1997) indicated that vicariance alone is sufficient to explain the current distribution of winter wren clades, and no dispersal has to be invoked. According to DIVA reconstruction, the ancestral population of winter wrens was widespread across the Holarctic. The first divergence resulted from western Nearctic birds separating from the Holarctic ancestor, presumably because they were isolated in a southern area of western North America at a time when Holarctic populations of wrens were still exchanging genes across Beringia (figure 1). Consistent with this reconstruction of their early history is the fact that the songs of European, Japanese and eastern North American wrens are much more similar to each other than any of them is to the songs of winter wrens from Washington and Oregon (Kroodsmas & Momose

1991). This first divergence was followed by the separation of eastern Nearctic and Palaeartic birds. This second split is likely to have involved isolation across Beringia because prior to major Middle Pleistocene glaciations, eastern Nearctic wrens could have occupied an even larger portion of the northern Nearctic than they do to date. Within the Palaeartic, the first divergence was between the wrens of eastern and western Eurasia. Eastern Palaeartic birds diverged into east Asian and central Asian (represented by a single individual from Nepal in our study) clades, and western Palaeartic birds diverged into Caucasian and European clades (figure 1).

4. DISCUSSION

Our analysis identified six mtDNA clades of winter wren. This differentiation was geographically structured and clades corresponded to large geographical regions: eastern Asia, Nepal (central Asia), Caucasus, Europe, eastern Nearctic and western Nearctic. The differentiation among the six clades was 3.0–8.8% of ML nucleotide divergence. These deep divergences argue for recognition of the six clades as evolutionarily significant units (Moritz 1994), or even species. Strong differentiation of winter wren clades and the relationships among them are also supported by differences in their vocalization (Kroodsmas & Momose 1991).

Virtually all sequence variation in winter wren was explained by differences among the six regions (96%). Differences among localities or subspecies within the six regions explained only 1% of sequence variation. Subspecies were not reciprocally monophyletic and many shared haplotypes. Thus, subspecific divisions in winter wren do not reflect the species' evolutionary history, as found for many other birds (Zink 2004). Unlike in most other cases, however, the numerous subspecific divisions in winter wren have obscured the recognition of deep, species-level, evolutionary divisions.

Although an ancestral form could have existed since the Middle Miocene (13 MYBP), proliferation of winter wren into the six clades coincided with the Early and Middle Pleistocene glaciations. Late Pleistocene glaciations did not produce distinct clades, but presumably contributed to self-pruning of the ND2 haplotype tree. In the localities that were free of ice during the last glaciation, population

age does not exceed 52 000 years (middle of the previous interstadial), which is an order of magnitude younger than the age of the most recent divergence among the clades (540 000 years).

Mismatch distributions (figure 1) suggested recently expanding populations, except for the Honshu population, whose mismatch distribution was significantly different from that expected for a recently expanding population despite its unimodal shape and low mean. Northward expansion during the current interstadial could have resulted in fragmentation of the southern parts of the species' range and in a loss of genetic variability in Japan (Hewitt 2000). A recent loss of genetic variability can produce a unimodal mismatch distribution similar to that of rapidly expanded populations. The slope of the distribution plot in declining populations, however, is steeper, especially on the right side of the graph, than in recently expanded populations (Rogers & Harpending 1992). Although the Japanese winter wren population appears stable, our samples are from central Honshu, where wrens are on the periphery of the core population to the north, and where they have low breeding success (K. Ueda, personal communication). The loss of genetic variability in Japanese winter wren is also supported by the fact that the population from Daito Islands (800 km south of Kyushu) recently became extinct and the population from Izu Islands (just southeast of Honshu) is endangered (Ministry of the Environment of Japan 1998). Furthermore, nucleotide diversity in Sakhalin Island, to the north of Hokkaido, is 2.8 times greater than in central Honshu. Declines in Japanese populations of some other temperate species are also known. For example, in the 1970s Asian rosy-finch (*Leucosticte arctoa*) probably bred in Hokkaido, where adults and fledglings were observed during the breeding season, but it no longer appears to breed in Japan (Brazil 1991).

The current distribution of winter wren clades can be explained by a sequence of five vicariant events (figure 1) that were probably caused by Early and Middle Pleistocene glaciations. These glaciations began periodic separations between the Nearctic and Palaearctic across Beringia, and between regions within these continents. Winter wrens could have had over 10 Myr to acquire a wide Holarctic distribution during the Late Miocene and Pliocene when the continents were connected across Beringia and many animals dispersed from one continent to the other. It is difficult to reconstruct the routes by which winter wren may have spread throughout the Holarctic because habitat distributions have changed dramatically since the Miocene. For example, mountain ranges currently inhabited by some clades (e.g. Caucasus, Himalayas) developed during the Miocene. However, colonization routes probably had little effect on differentiation of the six clades because these clades seem to have differentiated from a single, undifferentiated Holarctic ancestor.

While the six clades discovered with our samples can readily be explained by vicariance, dispersal may still be needed to explain some aspects of the distribution of winter wrens. Most obvious are the populations of winter wrens currently inhabiting Iceland and other islands of the North Atlantic. These islands could have been colonized by dispersal from either Europe or the eastern Nearctic.

Samples from these islands, together with larger samples from eastern North America and Europe, will be needed to determine the source populations for these colonizations.

The winter wren appears to differ considerably in phylogeographic structure from the other three Holarctic avian taxa studied to date. It is strongly differentiated within and between continents and differences among clades are much greater (almost threefold) than in the dunlin (Wenink *et al.* 1996), the common raven (Omland *et al.* 2000) and the three-toed woodpecker (Zink *et al.* 2002). Additional detailed population genetics studies of widely distributed Holarctic taxa are needed to elucidate whether these differences among taxa in the degree of intrataxon differentiation are related to their ecology and dispersal ability or to their demographic history.

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